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Date: <u>1.16.01</u> Express Mail Label No. <u>EL 762340309 US</u>
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Inventor(s): Qian Huang, Joan F.L. Richmond, Bryan K. Cho, Deborah
Palliser, Jianzhu Chen, Herman N. Eisen and Richard A. Young
Attorney's Docket No.: 0399.2006-003

In Vivo CTL Elicitation by Heat Shock Protein Fusion Proteins Maps to a Discrete
Domain and is CD4⁺ T Cell-Independent

RELATED APPLICATION(S)

This application is a continuation of International Application No.

- 5 PCT/US00/32831, which designated the United States and was filed on December 1, 2000, which will be published in English, in approximately 18 months from the priority date (June 2000), and which claims the benefit of U.S. Provisional Application No. 60/176,143, filed on January 14, 2000. The entire teachings of the above application(s) are incorporated herein by reference.

10 GOVERNMENT SUPPORT

The invention was supported, in part, by National Institutes of Health (NIH) training grant 5T32-AI-07463, NIH Cancer Center core grant CA-14051 and NIH research grants AI44476 and AI44478. The Government has certain rights in the invention.

15 BACKGROUND OF THE INVENTION

- When injected into an individual with diverse adjuvants, protein antigens usually stimulate the production of high-affinity IgG antibodies, indicating that they activate CD4 T helper cells, as well as B cells. These procedures generally fail, however, to elicit effective CD8 T cell responses. The reason, according to current views, is that the
- 20 short peptides needed, in association with MHC class I molecules, to stimulate CD8 T

cells arise from proteolytic cleavage of cytosolic proteins. Since injected protein antigens are generally unable to cross cellular lipid membranes, they fail to gain entry to the proper cytosolic "MHC class I processing pathway" and are thus unable to stimulate the production of CD8 T cells. Although there is evidence for alternative cellular pathways for processing some exogenous proteins to form peptide MHC class I complexes (Sigal, L.J., *et al.*, *Nature*, 398:77-80 (1999) and Gromme, M., *et al.*, *Proc. Natl. Acad. Sci. USA*, 96:10326-10331 (1999)) it remains generally true that protein antigens normally fail to stimulate significant CD8 CTL responses (Rock, K., *Today*, 17:131-137 (1996)).

- 5 There is now substantial evidence that heat shock proteins (hsps) isolated from tumors can be used as adjuvant-free anti-tumor vaccines in animals; hsp70 and the distantly related chaperones gp96 and calreticulin share this immunostimulatory activity (Udono, H. and Srivastava, P.K., *J. Exp. Med.*, 178:1391-1396 (1993); Udono, H., *et al.*, *Proc. Natl. Acad. Sci. USA*, 91:3077-3081 (1994); Suto, R. and Srivastava, P.K.,
- 15 *Science*, 269:1585-1588 (1995); Blanchere, N.E., *et al.*, *J. Exp. Med.*, 186:1315-1322 (1997); Tamura, Y., *et al.*, *Science*, 278:117-120 (1997) and Nair, S., *et al.*, *J. Immunol.*, 162:6426-6432 (1999)). The fusion of large polypeptides (80-110 amino acids in length) to mycobacterial hsp70 (TBhsp70) creates potent immunogens that can elicit MHC class I-restricted, CD8⁺ cytotoxic T cell responses sufficient to mediate rejection
- 20 of tumors expressing the fusion partner (Suzue, K., *et al.*, *Proc. Natl. Acad. Sci. USA*, 94:13146-13151 (1997)).

- The means by which soluble hsp70 fusion proteins stimulate CD8 cytotoxic T cell (CTL) responses are unknown. Among the possible mechanisms are: 1) strong hsp-specific CD4⁺ helper cell responses that enhance what might otherwise be a
- 25 minimal response to the soluble proteins (Barrios, C., *et al.*, *Eur. J. Immunol.*, 22:1365-1372 (1992); Suzue, K. and Young, R.A., *J. Immunol.*, 156:873-876 (1996); Horwitz, M.S., *et al.*, *Nature Med.*, 4:781-785 (1998) and Könen-Waisman, S., *et al.*, *J. Infect. Dis.*, 179:403-413 (1999)); and 2) chaperone function of hsps delivers the fusion protein to intracellular compartments of antigen-presenting cells for processing into short
- 30 peptides and loading onto MHC class I (Young, R.A., *Ann. Rev. Immunol.*, 8:401-420

(1990) and Schild, H., *et al.*, *Curr. Opinion Imm.*, 11:109-113 (1999)). An understanding of the ability of hsp70 to stimulate CD8⁺ CTL responses is needed to provide for more effective immunological prophylaxis and therapy for cancer and infectious diseases caused by intracellular pathogens.

5 SUMMARY OF THE INVENTION

The present invention is based on the discovery that a heat shock protein (hsp; hsps are also known in the art as stress proteins), or a discrete domain thereof, that is joined to a heterologous molecule can produce a CD8⁺ cytotoxic (cytolytic) lymphocyte (CTL) response in a host to which it is administered. The domain can be, for example,
10 about half (*e.g.*, 40, 45, 50, 55, or 60%) of the adenosinetriphosphate (ATP) binding domain of an hsp. Moreover, the response is independent of CD4⁺ CTLs. Accordingly, the invention features compositions that include an hsp, or all or a portion of an hsp ATP binding domain, joined to a heterologous molecule and methods of inducing a CD8⁺ CTL response to a molecule in an individual (*e.g.*, a patient, such as a human
15 patient, who has a deficiency of CD4⁺ T cells) by administering that composition to the individual. The method can be used to treat a patient who has an acquired immune deficiency syndrome (AIDS) by, for example, administering to the patient an hsp, or a portion of an ATP binding domain of an hsp, that is joined to a molecule associated with a human immunodeficiency virus (HIV), such as an HIV antigen.

20 The invention has numerous advantages. For example, the compositions and methods described herein provide for highly effective CD8⁺ CTL responses. These responses are useful in treating (*i.e.*, preventing or reducing the length or severity of symptoms associated with a disease process or preventing or attenuating the cellular events through which those symptoms are made manifest; treatment may be effective
25 without completely eradicating all symptoms) diseases that are caused by or otherwise associated with intracellular pathogens. Diseases or conditions that are characterized by a deficiency (or complete lack of) CD4⁺ T cells are particularly amenable to treatment.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a graph of effector cell to target cell ratio (E:T) versus % specific lysis illustrating OVA-specific CTL elicited by immunization with OVA.TBhsp70 fusion protein without adjuvant in the splenocytes from wild-type C57/BL/6 mice.

5 Figure 1B is a graph of E:T versus % specific lysis illustrating OVA-specific CTL elicited by immunization with OVA.TBhsp70 fusion protein without adjuvant in the splenocytes from CD4⁺ knockout mice (CD4 -/- mice).

Figure 1C is a graph of E:T versus % specific lysis illustrating OVA-specific CTL elicited by immunization with OVA.TBhsp70 fusion protein without adjuvant in
10 the splenocytes from mice which have very few CD8⁺ T cells (β 2m -/- mice).

Figure 2A is a graph of E:T versus % specific lysis illustrating murine hsp70 fusion protein elicits CTL responses in wild-type C57BL/6 mice.

Figure 2B is a graph of E:T versus % specific lysis illustrating murine hsp70 fusion protein elicits CTL responses in CD4 -/- mice.

15 Figure 3 is an illustration of the domains of the full length TBhsp70 which were separated into four segments I, II, III and IV and fused to C-terminal of OVA to make OVA.TBhsp70 fusion proteins; the numbers beneath each segment refer to the amino acid positions in TBhsp70.

Figure 4 is a graph of E:T versus % specific lysis illustrating OVA-specific T
20 cell responses in mice immunized with OVA fused to domains of TBhsp70; splenocyte cultures from mice primed with OVA (Δ), OVA.TBhsp70 (\blacksquare), OVA.TBhsp70 I (∇), II (\blacklozenge), III (X) and IV (+) were used as effector cells in the cytotoxicity assay.

Figure 5A is the P1 peptide amino acid sequence. When liberated from P1,
SIYRYYYGL (SEQ ID NO: 1) (boldface) binds to K^b to form the peptide-MHC complex
25 recognized by the 2C TCR. In P1, SIYRYYYGL (SEQ ID NO: 1) is flanked 5' and 3' by sequences that lie immediately upstream and downstream, respectively, of peptide bonds that are cleaved (see arrows) in murine cells to liberate naturally occurring peptides (SIINFEKL (SEQ ID NO: 2) from ovalbumin (Ova) and LSPFPFDL (SEQ ID

NO: 3) from α -ketoglutaraldehyde dehydrogenase (α KG) (Falk, K., *et al.*, *Eur. J. Immunol.*, 22:1323-1326 (1992); Ukada, K., *et al.*, *J. Immunol.*, 157:670-678 (1996)).

Figure 5B is a diagram of the hsp65-P1 fusion protein, showing P1 at the C-terminus of hsp65.

5 Figure 5C are histograms which provide evidence that P1 and hsp65-P1 are processed intracellularly to yield the SYRGL (SEQ ID NO: 4) octapeptide. 48 hr after transfection with mammalian expression vectors (VR1055 and pCINeo), containing sequences that encode P1 and hsp65-P1, respectively, EL4 cells were incubated for 18 hr with an equal number of naive 2C T cells. Histograms show the percentage of live,
10 2C⁺CD8⁺ cells that were stimulated to upregulate the activation marker CD69. The responses of these naive T cells to control EL4 cells, transfected with the empty (vector) plasmids, are shown as shaded histograms.

Figure 5D is a graph showing normal C57BL/6 mice have T cells that can recognize the SYRGL-K^b complex. A CD8⁺ T cell line, derived from C57BL/6 mice
15 immunized with the SYRGL (SEQ ID NO: 4) peptide in adjuvant, specifically lysed T2-K^b target cells in a peptide-dependent manner. A highly cytolytic long-term cultured 2C CTL clone (L3.100) is shown for comparison.

Figure 6A is a graph showing CD8 CTL that recognize the SYRGL-K^b complex are produced in C57BL/6 mice injected with hsp65-P1 in PBS but not in those injected
20 similarly with equimolar amounts of various controls (a mixture of P1 and hsp65, the SYRGL (SEQ ID NO: 4) octapeptide, the P1 polypeptide itself, hsp65 itself).

Figure 6B is a graph showing SYRGL-specific CTL in mice injected with a various amounts of hsp65-P1, 0.015-1.5 nmoles (1-100 μ g) or a control fusion protein in which P1 is linked to the C-terminus of a maltose-binding protein (Mal-P1, 80 μ g); lysis
25 of T2-K^b target cells in the absence of added SYRGL (SEQ ID NO: 4) peptide is indicated by unfilled symbols.

Figure 6C are graphs showing depletion of CD8⁺ T cells eliminates the SYRGL-specific CTL produced by mice injected with hsp65-P1. Lymph node and spleen cells from C57BL/6 mice immunized with 1.5 nmoles of hsp65-P1 or Mal-P1 were cultured
30 for 6 days and then depleted of CD8 T cells by magnetic sorting. The untreated, CD8-

depleted, and CD8-enriched populations (30%, 1%, 90%, CD8⁺ T cells respectively) were analyzed in a 4 hr cytolytic assay; lysis of T2-K^b target cells in the absence of added SYRGL peptide is indicated by unfilled symbols.

Figure 7 is a graph showing ⁵¹Cr-labeled splenic dendritic cells (spl dc), bone-marrow derived dendritic cells (bm-dc), or purified macrophages (mø), isolated from peritoneal lavage, all from B6 (H-2^b) mice were incubated for 4 hr with a 2C CTL clone (L3.100: see Fig. 5D) and various concentrations of the SYRGL octapeptide. CTL target cell ratio (E:T)=5:1. Unfilled symbols show lysis when the control fusion protein (Mal-P1) was used in place of hsp65-P1.

Figure 8A illustrates that splenic dendritic cells and peritoneal lavage macrophages were purified by magnetic sorting and incubated for 18-24 hr with equimolar concentrations of hsp65-P1 or Mal-P1 before adding naive 2C T cells. Expression of the activation marker CD69, Hsp65-P1 or Mal-P1 were added to purified splenic dendritic cells, macrophages, or to media alone ("no APC") at 15nM (~1 µg/ml). After 24 hr, purified naive 2C T cells were added (T cell:APC ratio of 1:1), and 18 hr later cells were analyzed for CD69, gating on propidium iodide-negative 2C⁺CD8⁺ cells. The percentage of 2C T cells with increased expression of CD69⁺ is indicated.

Figure 8B illustrates that splenic dendritic cells and peritoneal lavage macrophages were purified by magnetic sorting and incubated for 18-24 hr with equimolar concentrations of hsp65-P1 or unmodified hsp65 before adding naive 2C T cells. Dendritic cells or macrophages were incubated with hsp65-P1 or hsp65 before adding the naive 2C T cells and incubation was continued for an additional 18 hr (IL-2 assay) or 60 hr (proliferation assay) or 48 hr (IFN-γ assay). "No Ag" means the dendritic cells and 2C T cells were present but hsp65-P1 and hsp65 were absent: "No T" cells means the hsp65-P1 was present but the 2C T cells were omitted.

Figure 8C is a graph illustrating inhibition of responses by a clonotypic monoclonal antibody to the 2C TCR (1B2). Bone marrow derived dendritic cells were incubated with 10µg/ml hsp65-P1 overnight. Equal numbers of naive 2C T cells were then added in the presence or absence of 1B2 Fab fragments (25µg/ml). After an additional 18 hr, cells and supernatants were analyzed, respectively, for CD69

expression (left panel) and IL-2 production (3H-thymidine incorporation by IL-2-responsive HT2 cells, right panel).

Figure 9A is a pair of graphs comparing dendritic cells' and macrophages' ability to stimulate T cell responses at limiting antigen dose in vitro. Fresh splenic dendritic cells or macrophages were incubated with various concentrations of hsp65-P1 or Mal-P1 fusion proteins for about 18 hr before adding purified naive 2C T cells (see Figs. 8A, 8B). Supernatants were sampled 18 hr later to determine IL-2 levels (upper panel). ³H-thymidine was added at 48 hr and cells were harvested after an additional 18 hr to assess T cell proliferation (lower panel).

Figure 9B is a pair of graphs illustrating the behavior of hsp65 fusion protein-activated dendritic cells in vivo. Activation of dendritic cells in vivo. Myeloid dendritic cells from lymph nodes draining a subcutaneous site where hsp654-P1 was injected 24 hr previously show increased expression of MHC-1 (Kb) (lower panel) compared to myeloid dendritic cells from lymph nodes draining an uninjected site ("no treatment", upper panel).

Figure 9C is a trio of graphs illustrating the behavior of hsp65 fusion protein-activated dendritic cells in vivo. Dendritic cells activated with a noncognate hsp fusion protein (hsp65-NP) and pulsed with 10^{-9} M SYRGL (SEQ ID NO: 4) peptide are more effective than nonactivated, similarly pulsed dendritic cells in stimulating naive T cells in vivo. 8×10^5 dendritic cells were injected into a hind footpad of normal B6 mice that had been injected (iv) with 2×10^6 naive 2C TCR+ cells (from 2C TCR transgenic mice RAG-deficient mice). 24 hrs after the footpad injection, 2C CD8⁺ T cells in the draining popliteal lymph node were examined for CD69 expression. Frequency of CD69⁺ 2C CD8⁺ T cells in a lymph node draining the site where activated (control) dendritic cells (not pulsed with peptide) were injected (upper panel), or where SYRGL peptide-pulsed (1×10^{-9} M) unactivated dendritic cells or activated dendritic cells were injected (middle panel and lower panel, respectively). Percentages of CD69⁺ 2C cells are shown. Geometric means fluorescence values for MHC-1 (K^b) on dendritic cells that had been incubated, prior to footpad injection, with or without hsp65-NP were 379 and 97, respectively.

Figure 10A is a pair of graphs of dendritic cell MHC class I expression plotted as a function of protein concentration of the added hsp fusion proteins and control proteins. Upper panel; dendritic cells from C57BL/6 mice. Lower panel; dendritic cells from C3H mice. Purified bone marrow derived dendritic cells were incubated for 24 hr with various concentrations of hsp65-P1 or other hsp65 fusion proteins, having as fusion partners influenza virus nucleoprotein (hsp65-NP) or human papilloma virus, type 16, E7 subunit (hsp65-E7 preparations #1 and #2) or with controls (hsp65 alone, P1 alone, E7 alone, an anti-TNP IgG antibody). MHC Class I protein levels on the dendritic cells were then determined by flow cytometry by gating on propidium iodide-negative CD11c⁺ cells and using the Y3 antibody which recognizes both H-2^b (K^b) and H-2^k MHC class I. MHC class I levels are shown as geometric mean fluorescence; the levels on untreated dendritic cells are represented by a dashed horizontal line.

Figure 10B is a pair of graphs of the dendritic cell MHC class I expression values from Figure 10A and are plotted as a function of endotoxin concentration (calculated from the endotoxin levels present in the added hsp fusion proteins and other proteins). Upper panel; dendritic cells from C57BL/6 mice. Lower panel: dendritic cells from C3H mice.

Figure 10C is a graph showing that Hsp65-P1 stimulates production of CTL (anti-SYRGL) in CD4-deficient (CD4^{-/-}) mice. As in Fig. 6A-6C the mice were injected s.c. twice, one wk apart, with 100μg of hsp65-P1 or Mal-P1 in PBS. One wk following the second injection, cells from spleen and draining lymph nodes were pooled and restimulated with 1μM SYRGL (SEQ ID NO: 4) peptide without addition of exogenous cytokines. Six days later the cells were used as effectors in a standard 4 hr cytolytic assay at various E:T ratios using ⁵¹Cr-labeled T2-K^b cells as targets in the presence of 1μM SYRGL (SEQ ID NO: 4). Lysis of T2-K^b cells in absence of SYRGL (SEQ ID NO: 4) is shown by unfilled symbols.

Figure 11 is the nucleotide (cDNA) (SEQ ID NO: 5) and amino acid (SEQ ID NO: 6) sequences of *Mycobacterium tuberculosis* hsp70 (TBhsp70) wherein segment II (nucleotides 481-1110; amino acids 161-370) is highlighted.

Figure 12 is the nucleotide (SEQ ID NO: 7) and amino acid (SEQ ID NO: 8) sequences of segment II of TBhsp70.

Figures 13A-13B are the nucleotide (SEQ ID NO: 9) and amino acid (SEQ ID NO: 10) sequences of murine hsp70 wherein segment II (nucleotides 568-1194; amino acids 190-398) is highlighted.

Figure 14 is the nucleotide (SEQ ID NO: 11) and amino acid (SEQ ID NO: 12) sequences of segment II of murine hsp70.

DETAILED DESCRIPTION OF THE INVENTION

An immunological response to a molecule that, notably, includes a CD8⁺ CTL response, can be evoked in an individual by administering to that individual either an hsp joined to that molecule or a portion of an ATP binding domain of an hsp joined to that molecule (the molecule being virtually any biological substance, naturally- or nonnaturally-occurring with the exception of a portion of a stress protein). The CD8⁺ CTL response can be evoked in an individual who has a deficiency of CD4⁺ T cells (*i.e.* a CD4⁺ T cell count considered by any routinely used medical standard to be physiologically abnormal). Physicians and others having ordinary skill in the art can identify such individuals, which include patients infected with HIV. Accordingly, patients who are infected with HIV, or at risk of becoming so, can be treated with either an hsp joined to an HIV antigen (*e.g.*, p24 or gp41) or a portion of an ATP binding domain of an hsp joined to an HIV antigen (*e.g.*, p24 or gp41). Physicians and others having ordinary skill in the art can recognize and use other molecules associated with the HIV.

Heat shock proteins useful in the present invention are those that have an ATP binding domain and those that, when administered to an individual, induce a CD8⁺ T cell response to a molecule to which they are joined. Full length hsps (*e.g.*, hsp70 and hsp65) can be used, as can the ATP binding domains of hsps (or portions thereof). For example, the hsp moiety joined to the molecule can be the amino-terminal portion of the ATP binding domain. For example, the hsp moiety joined to the molecule can include or consist of about half of the ATP binding domain. For example, the hsp moiety can

include or consist of about 25 to about 365 consecutive amino acid residues (*e.g.* 25-350, 50-300, 110-275, 125-250, 130-225, 135-200, 150-200, 170-190, or 100-200 residues) of the ATP binding domain. More specifically, the hsp moiety can include or consist of amino acid residues 161-370 of *Mycobacterium tuberculosis* hsp70 or amino acid residues 190-398 of murine hsp70. Portions of hsp65 that are homologous to segment II of hsp70 (*e.g.* mycobacterial hsp65 such as *Mycobacterium bovis* BCG; mammalian hsp65, such as murine, canine, porcine, equine or human hsp65) can be used as described herein.

Those of ordinary skill in the art are well able to identify hsps and ATP binding domains within those proteins. Moreover, those artisans can make substitutions, if desired, in the sequences of these proteins or their domains that do not substantially reduce the abilities of those proteins or their domains to effectively induce CD8⁺ T cell responses. Amino acid substitutions can be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, or the amphipathic nature of the residues involved. For example, the nonpolar (hydrophobic) amino acid residues alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine can be substituted one for another; polar neutral amino acid residues such as glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine can be substituted one for another; positively charged (basic) amino acid residues such as arginine, lysine, and histidine can be substituted one for another; and negatively charged (acidic) amino acid residues such as aspartic acid and glutamic acid can be substituted one for another. For example, the hsp moiety used as described herein can include 1-25% conservative amino acid substitutions.

Any hsp or any portion of the hsp ATP binding domain can be purified from natural sources, recombinantly produced, or chemically synthesized. For example, an hsp or a portion thereof can be obtained from mycobacteria (*e.g.*, *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium leprae*, or *Mycobacterium smegmatis*), mammals (*e.g.* a murine, canine, porcine, equine, or human), fungi, parasite, or bacteria. Methods for recombinantly producing hsps or portions thereof are

also well known (production in bacteria such as *E. coli*) are described herein. In addition, the hsp or the portion thereof can be obtained from a commercial supplier.

Molecules useful in the present methods include any molecule against which a CD4⁺ independent immune response is desired. A "molecule" includes, but is not limited to, proteins or fragments thereof (*e.g.*, proteolytic fragments), peptides (*e.g.*, synthetic peptides or polypeptides), antigens, glycoproteins, carbohydrates (*e.g.*, polysaccharides and oligosaccharides), lipids, glycolipids, DNA (*e.g.*, recombinant DNA), killed or attenuated whole organisms (*e.g.*, viruses, bacteria, mycobacteria, parasites or fungi) or portions thereof, toxins, toxoids or any other organic molecule.

Molecules useful in the present methods can be obtained from a variety of sources using techniques routinely practiced in the art. For example, the molecule can be obtained from pathogens or organisms such as bacteria, mycobacteria, viruses, fungi or parasites. While the molecule can be isolated (*e.g.*, purified or partially purified (*e.g.* physically separated from at least 50% of the biological substances with which it naturally associates), it can also be chemically synthesized, recombinantly produced, or purchased from a commercial supplier.

The hsp or portion thereof is "joined" to a molecule against which an immune response is desired. The term "joined" includes covalent attachment of the hsp, or a portion thereof, to the molecule. The conjugation can be carried out using techniques routinely practiced in the art (*e.g.*, by forming a covalent bond between the hsp, or the portion thereof, and the molecule or by reductive amination). The term "joined" also includes fused proteins, such as those created by recombinant techniques or chemical synthesis. The fusion protein can include the molecule fused to the amino-terminal region or the C-terminal region of the hsp, or the portion thereof.

The CD8⁺ CTL responses induced by the methods of the present invention can be used for prophylaxis and/or therapy of diseases or conditions, particularly those characterized by a lack or deficiency of CD4⁺ T cells. That is, the hsp or portion thereof joined to the molecule against which an immune response is desired can be administered to an individual either before or after a disease or condition is manifested and can result in prevention, amelioration, elimination or delay in the onset or

progression of the disease state. For example, the present invention can be used to prevent or treat an individual positive for human immunodeficiency virus (HIV) and the opportunistic infections associated with HIV. In one embodiment, the HIV positive individual is deficient in CD4⁺ T cells.

5 In the methods of the present invention, an effective amount of the hsp or portion thereof joined to the molecule against which an immune response is desired is administered to an individual (*e.g.*, mammal such as human). As used herein an “effective amount” is an amount that induces a CD4⁺ T cell independent immune response to the molecule in an individual. In a particular embodiment, an “effective
10 amount” is an amount such that when administered to an individual, it results in an enhanced CD8⁺ CTL response to the molecule relative to the CD8⁺ CTL response to the molecule in an individual to whom an effective amount was not administered. For example, an effective amount or dosage of the hsp or portion thereof joined to the molecule against which an immune response is desired is in the range of about 50
15 pmoles to about 5000pmole. In one embodiment, the dosage range is from about 80pmole to about 3500pmoles; in another embodiment, the dosage range is from about 100pmoles to about 2000pmoles; and in a further embodiment the dosage range is from about 120pmoles to about 1000pmoles. The appropriate dosage of hsp or portion thereof joined to the molecule against which an immune response is desired for each individual
20 will be determined by taking into consideration, for example, the particular hsp and/or molecule being administered, the type of individual to whom the composition is being administered, the age and size of the individual, the condition or disease being treated or prevented and the severity of the condition or disease. Those skilled in the art will be able to determine using no more than routine experimentation the appropriate dosage to
25 administer to an individual.

The hsp or portion thereof joined to the molecule against which the immune response is desired can be administered to the individual in a variety of ways. The routes include intradermal, transdermal, (*e.g.*, slow release polymers), intramuscular, intraperitoneal, intravenous, subcutaneous, oral, epidural and intranasal routes. Any
30 other convenient route of administration can be used, for example, infusion or bolus

injection, infusion of multiple injections over time, or absorption through epithelial or mucocutaneous linings. In addition, the hsp joined to the molecule can be administered with other components or biologically active agents, such as adjuvants, pharmaceutically acceptable surfactants (*e.g.*, glycerides), excipients, (*e.g.*, lactose),
5 liposomes, carriers, diluents and vehicles.

Further, in the embodiment in which the molecule is a protein (peptide), the hsp or portion thereof joined to the molecule can be administered by *in vivo* expression of polynucleotides encoding such into an individual. For example, the hsp or portion thereof and/or the molecule can be administered to an individual using a vector, wherein
10 the vector which includes the hsp or portion thereof joined to the molecule is administered under conditions in which the hsp or portion thereof and the molecule are expressed *in vivo*.

Several expression system vectors that can be used are available commercially or can be produced according to recombinant DNA and cell culture techniques. For
15 example, vector systems such as yeast or vaccinia virus expression systems, or virus vectors can be used in the methods and compositions of the present invention (Kaufman, R.J., *J. Meth. Cell and Molec. Biol.*, 2:221-236 (1990)). Other techniques using naked plasmids or DNA, and cloned genes encapsulated in targeted liposomes or in erythrocyte ghosts can be used to introduce the hsp or portion joined to the molecule
20 into the host (Friedman, T., *Science*, 244:1275-1281 (1991); Rabinovich, N.R., *et al.*, *Science*, 265:1410-1404 (1994)). The construction of expression vectors and the transfer of vectors and nucleic acids into various host cells can be accomplished using genetic engineering techniques, as described in manuals like *Molecular Cloning* and *Current Protocols in Molecular Biology*, which are incorporated by reference, or by
25 using commercially available kits (Sambrook, J. *et al.*, *Molecular Cloning*, Cold Spring Harbor Press, 1989; Ausubel, F.M., *et al.*, *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley-Interscience, 1989).

As demonstrated in Example 1, hsp70 fusion proteins elicit CD8⁺ CTL in the absence of CD4⁺ T lymphocytes and this function resides in a 200-amino acid segment
30 of TBhsp70, indicating that chaperone activity is not required. To gain insights into the

mechanisms by which soluble hsp fusions can elicit CD8⁺ CTL against the fusion partner, hsp70 was dissected to ascertain whether a particular hsp domain is necessary, and knockout mice were used to determine whether the fusion protein's immunogenicity is dependent on CD4⁺ T lymphocytes. It was found that the ability to elicit CD8⁺ CTL depends on a discrete 200-amino acid protein domain, indicating that the fusion protein's immunogenicity for CD8⁺ T cells does not require coupled chaperone function or peptide binding. Further, it was found that ovalbumin.hsp70 fusion protein elicited anti-ovalbumin CD8⁺ CTL about equally well in CD4 knockout and wild-type C57BL/6 mice, and also when the hsp70 was of mycobacterial (*Mycobacterium tuberculosis*) or murine (self) origin. The ability of hsp70 fusion proteins to elicit CD4-independent CTL responses indicates that hsp70 fusion proteins can be used for immunological prophylaxis and therapy against disease in CD4⁺ T cell deficient individuals.

As demonstrated in Example 2, a mycobacterial heat shock protein, 65kDa (hsp65), fused to a polypeptide (P1) that contains an octapeptide (SIYRYYYGL (SEQ ID NO: 1)) agonist for a particular T cell receptor (2C TCR) stimulated C57BL/6 mice, as well as CD4-deficient mice, to produce CD8⁺ cytolytic T lymphocytes (CTL) to the fusion partner's octapeptide. This and other hsp65 fusion proteins, but not native hsp65 itself, stimulated dendritic cells, in vitro and in vivo, to upregulate the levels of MHC (class I and II) and costimulatory (B7.2) molecules. The results provide a mechanism for the general finding that hsp fusion proteins, having fusion partners of widely differing lengths and sequences, elicit CD8 CTL to peptides from the fusion partners, without requiring exogenous adjuvants or the participation of CD4⁺ T cells.

When mycobacterial hsp fused with large protein fragments, termed fusion partners, are injected into mice in saline solution (PBS) without added adjuvants several of them were previously shown to stimulate the production of CD8 CTL that recognize short peptide epitopes (8-10 amino acids in length) that arose from the fusion partners. The fusion partners varied from about 80 to 110 amino acids in length and were derived from ovalbumin (Suzue, K., *et al.*, *Proc. Natl. Acad. Sci. USA*, 94:13146-13151 (1997)), influenza virus nucleoprotein (Anthony, L., *et al.*, *Vaccine*, 17:373-383 (1999)), and an entire protein subunit of a human papilloma virus (N.R. Chu, personal communication).

As described in Example 2, to explore the mechanisms that permit these hsp to be effective with such diverse fusion partners, and that enable the hsp fusion proteins to serve as effective immunogens for CD8 T cells without requiring adjuvants, the immunogenic activities of fusion proteins prepared from the 65 kDa hsp from

5 *Mycobacterium bovis*, BCG strain (here called hsp65) were studied.

The principal fusion partner used in Example 2 was a polypeptide that contains an octapeptide sequence, SIYRYYYGL (SEQ ID NO: 1) (hereafter called SYRGL (SEQ ID NO: 4), Udaka, K., *et al.*, *Cell*, 69:989-998 (1992)), which together with K^b serves as a potent stimulator of CD8 T cells having the TCR of a CTL clone called 2C (Kranz, D.,
10 *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:573-577 (1984)). This peptide was identified in a synthetic peptide library and, so far as is known, does not occur in nature. The use of various T cells that express the 2C TCR, particularly naive 2C T cells (Cho, B., *et al.*, *Proc. Natl. Acad. Sci. USA*, 96:2976-2981 (1999)), were relied on as specific probes to obtain evidence that i) dendritic cells are more effective than macrophages in
15 presenting the processed hsp fusion protein to naive CD8 T cells, ii) dendritic cells are stimulated directly by each of several hsp65 fusion proteins tested, but not by "native" hsp65 itself, to increase surface expression of MHC class I and II and costimulatory (B7.2) molecules, and iii) CD4 T cells are not required for the fusion protein's ability to elicit production of CD8 CTL *in vivo*. Taken together, the results described herein
20 indicate that diverse soluble heat shock fusion proteins, regardless of the length or sequence of the fusion partners, stimulate CD8 T cell responses to peptides derived from the fusion partners without requiring exogenous adjuvants. The findings are of particular interest in view of the need to develop protective vaccines against intracellular pathogens for which current immunization strategies are inadequate (e.g.,
25 against HIV-1, human papilloma virus, various herpes viruses, malaria).

EXEMPLIFICATION

Example 1 *In Vivo* CTL Elicitation by hsp70 Fusion Proteins Maps to a Discrete Domain and is CD4⁺ T Cell-Independent

Materials and Methods

Expression vectors:

All constructs used to produce OVA.hsp70 fusion proteins were made in the bacterial expression plasmid pKS11h (Suzue, K., *et al.*, *Proc. Natl. Acad. Sci. USA*, 94:13146-13151 (1997)). Fusion constructs, consisting of OVA fused to the N-terminus of various segments of hsp70, were inserted downstream of the histidine tag sequence. A portion of ovalbumin (amino acid 230-359, hereafter referred to as OVA) was amplified from pOV230 (McReynolds, L.A., *et al.*, *Gene*, 2:217-231 (1977)) by PCR using upstream primer oQH025 and the downstream primer oQH027. Functional and structural domains of TBhsp70 based on crystal structures of ATP domain of bovine hsc70 (Flaherty, K.M., *et al.*, *Nature*, 346:623-628 (1990)) and peptide-binding domain of *E. coli* DnaK (Zhu, X., *et al.*, *Science*, 272:1606-1614 (1996)) were used. The full-length TBhsp70 were separated into four segments I, II, III and IV. The full-length TBhsp70 and each segment were fused to C-terminal of OVA to make OVA.TBhsp70 fusion proteins. (The sequences of these and other PCR primers are listed at the end of the Methods and Materials section).

The OVA expression vector pQH07 was constructed by subcloning OVA into the NdeI and NheI sites of pKS11h. Full-length TBhsp70 and four truncated TBhsp70 segments I (aa 1-166), II (aa 161-370), III (aa 360-517) and IV (aa 510-625) were amplified from plasmid pY3111/8 (kind gift of W. Wu, StressGen Biotechnologies, Vancouver Canada). The upstream primer for full-length TBhsp70 and segment I is oQH001, and the downstream primers are oJR061 and oQH011, respectively. The upstream primers for TBhsp70 II, III and IV are oQH012, oQH014 and oQH106, respectively. The downstream primers are oQH013, oQH015 and oJR061, respectively. The plasmids pQH06, pQH08, pQH09, pQH10 and pQH11, which express OVA fused to TBhsp70, TBhsp70 segment I, segment II, segment III and segment IV respectively, were constructed by subcloning the full-length and truncated TBhsp70 PCR products into the BamHI and EcoRI sites of pQH07 (at the C-terminus of OVA). Murine hsp70.1 coding sequence (referred to here as mhsp70) was amplified from plasmid pmhsp70.1 by PCR using the upstream primer oJR102 and the downstream primer oJR103. Plasmid pQH12, expressing OVA.mhsp70 fusion protein, was created by subcloning

mhsp70 into the BamHI and EcoRI sites of pQH07. All plasmids were verified by sequencing in both directions with double-stranded DNA templates.

Recombinant protein purification:

OVA, OVA.TBhsp70, OVA.TBhsp70 II, OVA.TBhsp70 III, and OVA.TBhsp70 IV were induced in *E. coli* (BL21(DE3)pLysS) for 9 hours at 25° in the presence of 0.5-1 mM isopropyl thiogalactoside (IPTG) and were purified as soluble proteins. The mycobacterial segment I and murine hsp70 fusion proteins were induced in *E. coli* for 4 hours at 37° with 1 mM IPTG and purified from inclusion bodies and then refolded as previously described (Suzue, K., *et al.*, *Proc. Natl. Acad. Sci. USA*, 94:13146-13151 (1997) and Suzue, K. and Young, R.A., *J. Immunol.*, 156:873-876 (1996)). All proteins were purified using nitrilo-triacetic acid Ni⁺ column (Qiagen, Hilden Germany) and HiTrap-Q anion exchange chromatography (Pharmacia, Piscataway, NJ) as previously described (Suzue, K., *et al.*, *Proc. Natl. Acad. Sci. USA*, 94:13146-13151 (1997) and Suzue, K. and Young, R.A., *J. Immunol.*, 156:873-876 (1996)). Purity was assessed using 4-20% gradient SDS-PAGE gels stained with Coomassie Blue (Bio-Rad, Hercules CA). All proteins were dialyzed against phosphate-buffered saline (PBS), and sterile filtered at 0.2μM. Protein concentrations were measured by Lowry assay (Bio-Rad) and expressed in molar terms to allow simple comparison of proteins of differing molecular weights.

Mice and immunizations:

Six- to eight-week old female C57BL/6, CD4^{-/-} and β2m^{-/-} mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and Taconic Farms (Germantown, NY). Both knockout mice have C57BL/6 (H-2^b) genetic backgrounds. Groups of 3 to 4 mice were injected intraperitoneally (i.p.) with 120 pmoles of recombinant protein in PBS; a second injection was performed subcutaneously (s.c.) two weeks later. The mice were sacrificed 10 days after the boost and splenocytes within groups were pooled (Suzue, K., *et al.*, *Proc. Natl. Acad. Sci. USA*, 94:13146-13151 (1997)).

Cell line:

EG7-OVA cells were cultured as previously described (Suzue, K., *et al.*, *Proc. Natl. Acad. Sci. USA*, 94:13146-13151 (1997)). OVA-specific CTL elicited by immunization with OVA.TBhsp70 fusion protein without adjuvant were examined. The splenocytes

5 from mice immunized with OVA (Δ) or OVA.TBhsp70 (\blacksquare) were incubated with irradiated EG7-OVA cells for 6 days in the absence of added cytokines and then used as effector cells (E) in a standard 4 hour cytotoxicity assay. The ^{51}Cr -labeled target cells (T) were: T2-K^b (dashed line) and T2-K^b-pulsed with SIINFELK peptide (SEQ ID NO: 2) (solid line) at 33 $\mu\text{g/ml}$. Splenocytes from wild-type C57/BL/6 mice are shown in

10 Figure 1A; splenocytes from CD4 $^{-/-}$ mice are shown in Figure 1B; and splenocytes from $\beta 2\text{m}^{-/-}$ mice are shown in Figure 1C.

CTL assays:

CTL assays were performed as described (Suzue, K., *et al.*, *Proc. Natl. Acad. Sci. USA*, 94:13146-13151 (1997)). Splenocyte cultures from mice primed with OVA (Δ),

15 OVA.TBhsp70 (\blacksquare), OVA.TBhsp70 I (∇), II (\blacklozenge), III (X) and IV (+) were used as effector cells in the cytotoxicity assay (See Figure 4). Results shown are representative of experiments repeated two to five times.

PCR primers:

oQH025 (5'- GCAGTACTCATATGATCCTGGAGCTTCCATTG

20 CCAGTGGGACAATG-3') (SEQ ID NO: 13)

oQH027 (5'- CTCCGACCTCACCTACGACGTTTCGAGAGACTTC

TTAAAATTATCCGATCGCCTAGACCTAGT-3') (SEQ ID NO: 14)

oQH001 (5'- ATAGTACTGGATCCATGGCTCGTGCGGTCGGGATC

GACCTCGGG- 3') (SEQ ID NO: 15)

oJR061 (5'- GGAATTCCTATCTAGTCACTTGCCCTCCCGGCCGTC-3') (SEQ ID NO: 16)

oQH011 (5'- GTCGACGAATTCATCATCAGATTCGCTGCTCCTTCTCGCCC TTGTCGAG-3') (SEQ ID NO: 17)

5 oQH012 (5'- GTCGACGGATCCATGGAGAAGGAGCAGCGAATCCTGGT CTTGACTTG-3') (SEQ ID NO: 18)

oQH014 (5'- GTCGACGGATCCATGGTGAAAGACGTTCTGCTGCTTGATGTTACCCCG-3') (SEQ ID NO: 19)

10 oQH016 (5'- GTCGACGGATCCATGCGTAATCAAGCCGAGACATTGGTCTA CCAGACG-3') (SEQ ID NO: 20)

oQH013 (5'-GTCGACGAATTCATCACGGGGTAACATCAAGCAGCAGAA CGTCTTTCAC-3') (SEQ ID NO: 21)

oQH015 (5'-GTCGACGAATTCATCAGACCAATGTCTCGGCTTGATTACG AACATCGGC-3') (SEQ ID NO:22)

oJR102 (5'-TCTAGAGGATCCATGGCCAAGAACACGGCGATC-3') (SEQ ID NO: 23)

oJR103 (5'-TCTAGAGAATTCCTAATCCACCTCCTCGATGGTGGGTCC-3') (SEQ ID NO:24)

Results and Discussion

Previous studies demonstrated that soluble, adjuvant-free TBhsp70 fusion proteins elicit substantial immune responses, including CD8⁺ CTLs, in mice (Suzue, K., *et al.*, *Proc. Natl. Acad. Sci. USA*, 94:13146-13151 (1997) and Suzue, K. and Young, R.A., *J. Immunol.*, 156:873-876 (1996)). The basis for the effectiveness of hsp70 fusions is unclear as most soluble proteins do not elicit significant CD8⁺ T cell responses (reviewed in Braciale, T.J., *et al.*, *Immunol. Rev.*, 98:95-114 (1987), Jondal, M., *et al.*, *Immunity*, 5:295-302 (1996)). While there is evidence that the hsp moiety of mycobacterial hsp fusion proteins acts as an effective carrier in the classic sense, enhancing B cell responses to chemically conjugated pneumococcal polysaccharides (Könen-Waisman, S., *et al.*, *J. Infect. Dis.*, 179:403-413 (1999)) and malarial polypeptide (Barrios, C., *et al.*, *Eur. J. Immunol.*, 22:1365-1372 (1992)), carriers are not known to stimulate CTL production. It was reasonable to expect that hsp70 fusion proteins provide hsp70-specific cognate CD4⁺ T cell help to OVA-specific CD8⁺ CTL by activating shared professional antigen presenting cells (APCs) as suggested by many, and demonstrated recently (Bennett, S.R.M., *et al.*, *Nature*, 393:478-480 (1998); Ridge, J.P., *et al.*, *Nature*, 393:474-478 (1998) and Schoenberger, S.P., *et al.*, *Nature*, 393:480-483 (1998)).

As described herein, this cognate help hypothesis was tested using CD4 deficient (knockout) mice (CD4^{-/-}). Wild-type C57BL/6, CD4^{-/-}, and β2m^{-/-} mice were each immunized with OVA or OVA.TBhsp70 fusion protein. As expected, immunization of wild-type mice with OVA.TBhsp70, but not OVA, generated CTL specific for the immunodominant epitope of OVA (SIINFEKL) (Figure 1A). The same results were obtained when the CD4^{-/-} mice were immunized with OVA.TBhsp70 (Figure 1B). β2m^{-/-} mice, which have very few CD8⁺ T cells, did not develop OVA-specific CTL after immunization with OVA.TBhsp70 or with OVA alone (Figure 1C).

Previous efforts to determine whether CD4⁺ T cell help is necessary for generation of CD8⁺ CTL have drawn differing conclusions. CD4 knockout mice exhibit a range of CD8⁺ CTL responses: CD4-dependent, weakly dependent, or independent. CTL responses to minor histocompatibility antigens (Ridge, J.P., *et al.*, *Nature*,

- 393:474-478 (1998), Guerder, S. and Matzinger, P., *J. Exp. Med.*, 176:553-564 (1992)) or to ovalbumin loaded into spleen cells (Bennett, S.R.M., *et al.*, *J. Exp. Med.*, 186:65-70 (1997)) are CD4- dependent. Some potent CD8⁺ T cell immunogens including viruses (Bachmann, M.F., *et al.*, *J. Immunol.*, 161:5791-5794 (1988)), such as
- 5 lymphocytic choriomeningitis virus (Leist, T.P., *et al.*, *J. Immunol.*, 138:2278-2281 (1987); Ahmed, R., *et al.*, *J. Virol.*, 62:2102-2106 (1988); Rahemtulla, A., *et al.*, *Nature*, 353:180-184 (1991) and von Herrath, M., *et al.*, *J. Virol.*, 70:1072-1079 (1996)), ectromelia virus (Buller, R.M., *et al.*, *Nature*, 328:77-79 (1987)) and some influenza virus subtypes (Wu, Y. and Liu, Y., *Curr. Biol.*, 4:499-505 (1994)), as well as
- 10 allogeneic cells (Krieger, N.R., *et al.*, *J. Exp. Med.*, 184:2013-2018 (1996)) elicit strong CD8⁺ T cell responses in wild-type and CD4^{-/-} mice. The similarity of CD8 CTL responses to OVA.TBhsp70 in CD4^{-/-} and wild-type mice suggests that hsp70 fusion proteins are relatively potent CD8⁺ CTL immunogens. A similar result, showing that CD4⁺ T cells are not required for the CD8⁺ CTL response elicited by another
- 15 mycobacterial heat shock fusion protein (hsp65 fused to a polypeptide containing an epitope for 2C CD8⁺ T cells) using CD4^{-/-} mice is described in Example 2. In addition, the ability of a non-homologous hsp, gp96, to elicit tumor rejection requires CD4⁺ T cells at tumor challenge, but not during priming with tumor-derived gp96 (Udono, H. and Srivastava, P.K., *Proc. Natl. Acad. Sci. USA*, 91:3077-3081 (1994)).
- 20 It has been proposed that the immunostimulatory effects of certain hsp fusion proteins may be due to the bacterial origin of the hsp moiety (Schild, H., *et al.*, *Curr. Opinon Imm.*, 11:109-113 (1999)). This possibility was examined by making OVA.hsp70 fusion proteins with the murine homologue of TBhsp70 (Hunt, C. and Calderwood, S., *Gene*, 87:199-204 (1990)), here referred to as mhsp70. Immunization
- 25 of wild-type C57BL/6 mice with OVA.mhsp70, but not OVA, elicited CTL responses equivalent to those generated by the TBhsp70 fusion protein (Figure 2A). The response to OVA.mhsp70 was also independent of CD4 (Figure 2B). Since a CD4⁺ T cell response to self (murine) hsp70 is unlikely, the effectiveness of the murine hsp70 fusion protein is in accord with the more direct evidence for CD4-independence obtained using
- 30 CD4^{-/-} mice (see above).

The ability of hsp fusion proteins to elicit CTLs against the fusion partner may be a consequence of the hsp moieties' chaperone activity, assuming that this activity is preserved in the fusion protein. To investigate this issue, TBhsp70 was divided into four linear segments and OVA and a glycine/serine linker were fused to the amino-

5 terminus of each segment, creating OVA.TBhsp70s I-IV (Figure 3). Each segment corresponds to a distinct structural domain of hsp70 as described by Flaherty, K.M., *et al.*, *Nature*, 346:623-628 (1990) and Zhu, X., *et al.*, *Science*, 272:1606-1614 (1996). As shown in Figure 3A, the amino-terminal ATP-binding domain was divided into its two structural lobes: I (aa 1-160) and II (aa 161-362). The carboxy-terminal peptide-binding

10 domain was divided into a β -sandwich domain, III (aa 364-512), and an α -helical domain, IV (aa 512-625).

Six groups of three C57BL/6 mice were immunized with 120 pmoles of OVA, OVA.TBhsp70, and OVA fused to segments I, II, III and IV. CTL assays showed that splenocytes from mice immunized with OVA.TBhsp70 and OVA fused to segment II

15 lysed T2-K^b cells in the presence, but not absence, of the OVA K^b epitope, SIINFEKL (Figure 4). In contrast, cells from mice immunized with OVA and OVA fused to segments I, III and IV were ineffective, even at an E:T ratio of 80:1. Levels of cytolysis obtained with splenocytes from mice immunized with OVA.TBhsp70 and OVA fused to segment II were indistinguishable (Figure 4). These results show that half of the ATP-

20 binding domain of TBhsp70 (aa 161-362) is sufficient to stimulate substantial production of anti-OVA CTL response in the absence of adjuvant.

Since it is highly unlikely that segment II preserves chaperone activity we conclude that the ability of the fusion proteins to elicit CD8⁺ T cell does not depend on the hsp moieties' chaperone properties. The data described herein support a model in

25 which hsp70 bypasses the need for CD4⁺ help by directly or indirectly activating or affecting the maturation state of APCs such as dendritic cells in a manner similar to some viruses (Ruedl, C., *et al.*, *J. Exp. Med.*, 189:1875-1883 (1999)). According to this model, hsp70 fusion proteins likely activate few CD8⁺ T cells to release immunostimulatory cytokines in draining lymph nodes. These cytokines, in turn,

30 provide the help required to upregulate expression of costimulatory molecules on APCs

in the lymph node, leading to further CD8⁺ T cell activation (Ruedl, C., *et al.*, *J. Exp. Med.*, 189:1875-1883 (1999)). Recent studies demonstrate that exposure of macrophages to bacterial and human hsp60 (Chen, W., *et al.*, *J. Immunol.*, 162:3212-3210 (1999); Kol, A., *et al.*, *J. Clin. Invest.* 103:571-577 (1990)), murine hsp70 and
 5 gp96 (Suto, R. and Srivastava, P.K., *Science*, 269:1585-1588 (1995); Breloer, M., *et al.*, *J. Immunol.*, 162:3141-3147 (1999)) increases expression of adhesion molecules and cytokines.

The ability to hsp70 fusion proteins to elicit CTL responses in the absence of CD4⁺ cells indicates that hsp70 can be used as a vehicle for the development of
 10 prophylaxis and therapy of diseases or conditions characterized by a lack or deficiency of CD4⁺ cells, such as HIV-1 and its opportunistic infections. Infections by HIV and its simian cousin SIV can lead to a substantial reduction of CD4⁺ T cells, thereby crippling the host's immune response to HIV and other pathogens. This loss of CD4⁺ cells is thought to impair the development and maintenance of CD8⁺ CTL responses (Kalams,
 15 S.A., *et al.*, *J. Virol.*, 73:6715-6720 (1999)). Recent studies conclude that strong HIV-specific CTL responses are required to keep HIV-1 infection in check and to destroy HIV-infected cells (Harrer, T., *et al.*, *AIDS Res. Hum. Retro.*, 12:585-592 (1996); Harrer, T., *et al.*, *J. Immunol.*, 156:2616-2623 (1996); Yang, O.O., *et al.*, *J. Virol.*, 70:5799-5806 (1996); Yang, O.O., *et al.*, *J. Virol.*, 71:3120-3128 (1997); Matano, T., *et al.*, *J. Virol.*, 72:164-169 (1998) and Wagner, L., *et al.*, *Nature*, 391:908-911 (1998)).
 20

Example 2 Heat Shock Fusion Proteins Stimulate Dendritic Cells and Elicit Production of Cytolytic T Lymphocytes Without Requiring Participation of CD4 T Cells

Methods and Materials

25 Mice, CTL clones and cell lines

C57BL/6 (H-2^b), Cd4-deficient (CD4 tm1Mak, H-2^b), and C3H/HeJ mice (H-2^k) were obtained from The Jackson Laboratories (Bar Harbor, ME), maintained in barrier cages under specific pathogen free conditions, and immunized between 4- and 10-weeks of age. 2C TCR transgenic mice (H-2^b) contain the rearranged transgenes encoding the

$\alpha\beta$ TCR from a 2C CTL clone (Sha, W., *et al.*, *Nature*, 335:271-274 (1988)). 2C TCR transgenic mice deficient for the recombination activating gene-1 (termed 2C/RAG) (Manning, T., *et al.*, *J. Immunol.*, 159:4665-4675 (1997)) were used as a source of naive T cells for in vitro assays (Cho, B., *et al.*, *Proc. Natl. Acad. Sci. USA*, 96:2976-2981 (1999)). 2C CTL clone. L3.100, has been previously described (Sykulev, Y., *et al.*, *Immunity*, 9:475-483 (1998)). EL4 cells were obtained from the ATCC (Rockville, MD) and T2-K^b cells were a generous gift from Peter Creswell, Yale University.

Plasmids, peptides, and proteins

In the P1 polypeptide the sequences flanking the – and C- termini of the SYRGL octapeptide (Fig. 5A), from ovalbumin (ova251-257) and α -ketoglutaraldehyde dehydrogenase, respectively, were modified by addition of a lysine residue penultimate to the N-terminus (out of ubiquitination consideration) (Eisenlohr, L., *et al.*, *J. Exp. Med.*, 175:481-487 (1992); York, I.A. and Rock, K.L., *Annu. Rev. Immunol.*, 14:369-396 (1996)), and an isoleucine and a tyrosine residue were added at the – and C-termini for cloning purposes. Complementary oligonucleotides encoding P1 were synthesized and cloned into a mammalian expression vector VR1055 (Vical, San Diego, CA), and subsequently subcloned as an in-frame fusion at the 3' end of *M. bovis* BCG hsp65 gene (hsp65-P1) into the bacterial expression vector pET28A+ (Novagen, Madison, WI). The P1 sequence was also subcloned into the 3' end of the gene encoding *E. coli* maltose binding protein in pMAL-p2, using the pMAL protein fusion system (New England Biolabs, Beverly, MA), as well as into the mammalian expression vector pCIneo (Promega, Madison, WI). All hsp65 fusion proteins used in this example, as well as the unmodified hsp65, were produced as recombinant proteins in *E. coli*. They were purified under denaturing conditions from the soluble fraction of bacterial lysates and fractionated successively on butyl-Sepharose, Q-Sepharose (and Ni-Sepharose when applicable), and finally by dialysis against PBS. Mal-P1 was purified by amylose affinity chromatography (New England Biolabs, Beverly, MA).

SDS-PAGE analysis of purified hsp65-P1 revealed a major species at 67.5kDa, which was shown to be hsp65-P1 by Western analysis, using anti-mycobacterial hsp65

specific antibody (StressGen, Victoria, Canada), and by electrospray mass spectrometry (M.I.T. Biopolymer Laboratory). Mal-P1 was also subjected to amino acid analysis and SDS-PAGE to confirm molecular weight (48.1kDa) and purity. P1 and SYRGL peptides were synthesized by the MIT Biopolymers Laboratory. Protein concentrations were estimated by bicinchoninic acid or amino acid analyses and were expressed in molar terms to facilitate comparisons between proteins and polypeptides of differing molecular masses. Endotoxin concentrations of recombinant protein preparations were determined by the Limulus assay, using reagents and conditions according to Associates of Cape Cod (Falmouth, MA). Peptide concentrations were estimated by W/V or based on amino acid analyses.

Antibodies and flow cytometry

Flow Cytometry was carried out on a FACSCaliber, using CellQuest software (Becton Dickinson, Franklin Lakes, NJ). Unlabeled or FITC-, PE-, allophycocyanin- or biotin-labeled antibodies against CD69, CD4, CD8, CD11c, CD11b, GR.1, B7.2, B220 or MCH class I (H-2^b), as well as secondary antibodies and streptavidin labeled with allophycocyanin, or PE, were obtained from Pharmingen (San Diego, CA). 1B2, a clonotypic antibody that recognized 2C TCR (Kranz, D., *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:573-577 (1984)), was purified from the 1B2 hybridoma and biotinylated using biotinamidocaproate N-hydroxysuccinimide ester (Sigma, St. Louis, MO). The antibody, Y3, is cross-reactive with MCH class I from H-2^b (K^b) and H-2^k haplotypes. It is affinity purified from culture supernatants from the Y3 hybridoma (obtained from ATCC, Rockville, MD) and labeled with fluorescein using fluorescein isothiocyanate.

Generation of bone-marrow derived dendritic cells and isolation of antigen presenting cells and naive 2C T cells

To generate bone-marrow derived dendritic cells from C57BL/6 (or C3H) mice, bone marrow was flushed from the femur and tibia, red blood cells were lysed, and the remaining cells were cultured at 10⁶ cells/ml in RPMI 1640 medium (supplemented with 10% heat-inactivated fetal calf serum, 2mM L-glutamine, 10mM HEPES, 50 µm

β -mercaptoethanol, 100 U/ml penicillin and 100 μ g/ml streptomycin) containing 20 ng/ml murine GM-CSF (R&D Systems, Minneapolis, MN). The medium was replaced on days 2 and 4, and on day 6 the cells (immature dendritic cells) were harvested for use.

- 5 In vitro assays were performed with purified cell populations unless otherwise noted. Magnetic cell sorting (MACS) was carried out according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Dendritic cells (splenic or bone marrow-derived) were isolated by positive sorting using anti-CD11c antibody (purity ranged from 70-97%). Peritoneal lavage macrophages were purified by treating them with
- 10 biotinylated antibodies specific for CD11c, GR.1, and B220, followed by washing and incubating them with magnetic microbeads coated with anti-CD4 or anti-CD8 antibodies or with streptavidin and then passing them over a negative sorting column. Macrophage purity was typically >90%. For purification of 2C T cells from 2C/RAG transgenic mice, lymph node and spleen cells were coated with anti-CD8 magnetic
- 15 beads (an average of 2 beads per cell) and positively sorted as above (purity >93%). The purification procedures did not activate APC or T cells as shown by flow cytometry: APC showed no increase in B7.2, MHC class I, or cell diameter, and T cells showed no CD69 upregulation after 24 hr in culture. There was also no significant ^3H -thymidine incorporation by T cells after 48 hr incubation.

20 Cytolytic T cell assays

- Unless otherwise noted, ^{51}Cr -labeled T2-K^b cells were used as target cells. They were incubated with effector cells derived from either fusion protein-injected mice or from cultured 2C T cell clones for 4 hr in the presence or absence of SYRGL (1 μ M). Specific lysis was calculated as follows: [(experimental counts - spontaneous counts)
- 25 / (total counts - spontaneous counts)] x 100.

To assess the ability of various APC to process hsp65-P1, dendritic cells and macrophages were used as target cells. Each of these cell populations was purified by MACS and then ^{51}Cr -labeled for 1 hr at 37°C. The labeled cells were then incubated with hsp65-P1 together with the 2C CTL clone (L3.100) at a CTL: target cell (E:T)

ration of 5:1. Assays were performed in triplicate using 96-well round bottom plates and cell supernatants were counted in a γ spectrometer after 4 hr. Specific lysis was calculated as above.

Transient transfection and antigen processing assays

- 5 EL4 cells (5×10^6) were electroporated with 15 μ g of the parent plasmids or plasmids containing the genes for P1 (in VR1055) or hsp65-P1 (in pCIneo). 48 hr after transfection, the cells were subjected to centrifugation in Ficoll-Paque (Pharmacia Biotech., Piscataway, NJ) (2200rpm, 20 min) and 10^6 live cells were incubated with an equal number of splenocytes from naive 2C/RAG mice. After 18 hr the cells were
- 10 stained with 1B2, anti-CD69, and anti-CD8 antibodies (labeled with FITC, PE, and allophycocyanin, respectively) and 2C T cells were evaluated for upregulation of CD69 by flow cytometry, gating on propidium iodide-negative, 1B2⁺, CD8⁺ cells.

Naive 2C T cell responses to dendritic cells and macrophages and dendritic cell activation assays

- 15 Purified dendritic cells and macrophages were incubated with various concentrations of proteins or peptides in 96-well (5×10^4 cells/well) flat bottom plates for 24 hr at 37°C. The following day an equal number of purified naive 2C T cells were added to each well (final volume: 200 μ l for the 96-well plates, 600 μ l for the 48-well plates). After 18 hr., the 48-well plates were separated into i) cell pellets to analyze 2C
- 20 T cells for expression of the acute activation marker CD69 by flow cytometry, gating on propidium iodide-negative. 1B2⁺ CD8⁺ cells, and ii) cell supernatants to measure IL-2 secretion (in triplicate, using HT2 cells in a standard bioassay) (Watson, J., *J. Exp. Med.*, 150:1510-1519 (1979)). After 48 hr, the 96-well plates were assayed for IFN- γ secretion (using 50 μ l of cell supernatants and a capture ELISA assay (R&D Systems,
- 25 Minneapolis, MN), and for T cell proliferation (1 mCi ³H-thymidine (NEN, Boston, MA) was added per well and 16 hr later the cells were harvested to measure ³H-thymidine-incorporation). Where indicated, 1B2 Fab fragments were added to naive 2C T cells at a final concentration of 25 μ g/ml.

Immature bone marrow-derived dendritic cells (day 6 of culture) were purified by magnetic sorting ($>95\%$ CD11b⁺CD11c⁺) and incubated (2.5×10^5 cells/well in 96-well round bottomed plates) with various fusion proteins or control proteins. The following day, cells were analyzed by flow cytometry for expression of B7.2 and MHC class I and class II molecules, gating on propidium iodide-negative, CD11c⁺ cells.

RESULTS

Design and characterization of heat shock fusion protein hsp65-P1

As shown in Figs. 5A and 5B, the principal fusion protein used herein contains the polypeptide P1 fused to the C-terminus of hsp65. P1 includes the octapeptide, SYRGL, that behaves, in association with K^b, as a strong agonist for the TCR on 2C T cells (Sykulev, Y., *et al.*, *Immunity*, 4:565-571 (1996)). The sequences that flank the octapeptide in P1 were chosen because they correspond to those known to be effectively cleaved intracellularly in two unrelated proteins: ovalbumin (Falk, K., *et al.*, *Eur. J. Immunol.*, 22:1323-1326 (1992)) and α -ketoglutaraldehyde dehydrogenase (Udaka, K., *et al.*, *Cell*, 69:989-998 (1992), see arrows, Fig. 5A). To determine if the P1 polypeptide, alone or linked as a fusion partner to hsp65, could be cleaved intracellularly to liberate the SYRGL octapeptide, we transfected plasmids containing sequences for P1 or hsp65-P1 were transfected into EL4 cells (H-2^b). Because relatively few of the transiently transfected cells were expected to express P1 or hsp65-P1, the transected cell population was not used in cytolytic assays as targets for 2C CTL. Instead, their ability to stimulate naive 2C T cells were examined. As shown in Fig. 5C, 80-90% of these naive T cells were stimulated to express the acute activation marker CD69 in response to EL4 cells transfected with either the P1 or hsp65-P1 plasmids, while virtually none of the naive T cells were activated by cells transfected with the empty plasmids (vector, shaded histograms, Fig. 5C). These results indicate that in these transfected cells P1 and hsp65-P1 can be cleaved to release the octapeptide, which is then presented by K^b.

C57BL/6 mice produce SYRGL-specific CD8⁺ cytolytic T cells in response to hsp65-P1

Before immunizing mice with hsp65-P1, it was first ensured that CD8 T cells that can recognize the SYRGL octapeptide (SEQ ID NO: 4) are present in normal C57BL/6 (H-2^b) mice. The mice were therefore injected with SYRGL peptide (SEQ ID NO: 4) in adjuvant (TiterMaxGold), their spleen cells were maintained in culture for several weeks (see Methods) and subsequently tested in a standard cytotoxicity assay. As shown in Fig. 5D, the cell line's lysis of K^b target cells (T2-K^b) was SYRGL (SEQ ID NO: 4)-dependent, indicating the presence in these mice of T cells that can respond to SYRGL-K^b complexes.

To determine if the hsp65-P1 fusion protein could stimulate ("prime") anti-SYRGL CTL in vivo, normal C57BL/6 mice were injected subcutaneously (s.c.) with the fusion protein in saline without added adjuvants. Each mouse received two injections, one wk apart. 7 days after the 2nd injection cells from regional lymph nodes and spleen were restimulated in culture with SYRGL (1 μ M) in the absence of exogenous cytokines, and tested after 6 days for CTL activity in a 4 hr cytolytic assay, using ⁵¹-Cr labeled K^b target cells (T2-K^b; see Methods). Of 40 injected mice, 35 produced CTL whose lysis of the K^b target cells was SYRGL-dependent (see Fig. 6A for a representative response). C57BL/6 mice treated in exactly the same way with equimolar amounts of various controls (hsp65, P1, or a mixture of hsp65 and P1, or SYRGL alone), all failed to yield SYRGL-specific CTL (Fig. 6A). As little as 1 μ g (0.015 nmoles) of hsp65-P1 could elicit an anti-SYRGL CTL response. A control fusion protein, made by fusing the P1 sequence to the C-terminus of another bacterial protein chosen simply for ease of purification (the *E. coli* maltose binding protein), here called Mal-P1, was around 10-100 times less effective in these assays (Fig. 6B) and without any detectable effect in others (Figs. 7, 8A-8C, 9A-9C, 10A-10C). Removal of CD8 T cells by magnetic sorting showed that the cytolytic response to hsp65-P1 was due to CD8 T cells (Fig. 6C). These results demonstrate that hsp65-P1, without added adjuvants, can elicit a CD8 T cell response to the fusion partner.

Dendritic cells and macrophages differ in ability to serve as antigen-presenting cells for hsp65-P1

To identify antigen presenting cells (APC) that mediate in vivo CD8 T cell responses, purified preparations of APC from C57BL/6 mice (dendritic cells from spleen or bone marrow, and macrophages from peritoneal lavage) were tested for ability to present processed hsp65-P1 and serve as target cells in cytolytic assays, using a well-established SYRGL-K^b specific CTL clone (L3.100) as effectors. When dendritic cells and macrophages were ⁵¹Cr-labelled and incubated with hsp65-P1 for 4 hr, they were lysed effectively and to about the same extent (Fig. 7). No significant lysis was observed, however, when the control fusion protein Mal-P1 was used in place of hsp65-P1, suggesting that processing of hsp65-P1 by these APC was not due to indiscriminate extracellular proteolysis.

Cytolytic reactions with potent CTL clones, such as L3.100, can be exquisitely sensitive, detecting very few and probably as little as one cognate peptide-MHC complex per target cell (Sykulev, Y., *et al.*, *Immunity*, 4:565-571 (1996)). Therefore, a more discriminating assay in which dendritic cells and macrophages that had been incubated with hsp65-P1 were compared as APC for their ability to stimulate naive 2C T cells was used. As shown in Fig. 8A-8C, when the dendritic cells were incubated with hsp65-P1 overnight and then with naive 2C T cells, the naive T cells were stimulated to: i) express CD69, ii) proliferate, and iii) secrete IL-2 and IFN- γ . In contrast, the macrophage preparations stimulated none of these responses. (It may be that activated macrophages would have behaved differently, but we deliberately focused on non-activated macrophages and dendritic cells to stimulate conditions in the immunized animal were deliberately focused upon. The response elicited by dendritic cells could be inhibited by the clonotypic, anti-2C TCR, antibody (1B2; Fig. 8C), indicating that they were mediated by ligation of the 2C TCR. The requirement for the hsp65 moiety in the hsp65-P1 fusion protein is emphasized by the result that naive 2C T cells were stimulated to express CD69 by dendritic cells that had been incubated with hsp65-P1 but not by those that had been incubated with the control fusion protein Mal-P1 (Fig. 8A).

Incubation of dendritic cells with various controls (P1 alone, hsp65 alone, or a mixture of hsp65 + P1) in place of hsp65-P1 did not stimulate 2C T cells to secrete IFN- γ . However, of all the controls the P1 peptide was exceptional in that it exhibited some activity; with both dendritic cells and macrophages it stimulated CD69 expression and
 5 with dendritic cells, but not with macrophages, it induced proliferation and IL-2 secretion by the naive 2C T cells. It is likely that the P1 peptide itself is subject to proteolysis by these APC, particularly by dendritic cells, but whether extracellularly or in some intracellular compartment is not clear. Whatever the explanation, it should be noted that the P1 polypeptide did not stimulate CD8 CTL production in vivo under
 10 conditions where the hsp65-P1 fusion protein was consistently effective (Fig. 6A). In addition, as is shown later, P1 also failed to activate dendritic cells (see Figs. 10A-10C).

To examine the difference between dendritic cells and macrophages more closely, these cells were incubated with various concentrations of the fusion proteins and then evaluated their ability to stimulate naive 2C T cells. As shown in Fig. 9A, the
 15 naive cells proliferated and produced substantial amounts of IL-2 in response to dendritic cells that had been incubated with concentrations of hsp65-P1 in the 0.1- .01 μ M range. In contrast, the responses by naive cells were negligible when macrophages were used in place of dendritic cells or when the Mal-P1 control fusion protein was used at concentrations up to 1 μ M (Fig. 9A). Together, these data suggest that dendritic cells
 20 are more effective than macrophages in processing and presenting the octapeptide from hsp65-P1.

Heat shock fusion proteins stimulate dendritic cells directly

The distinctive ability of hsp65-P1 to stimulate naive 2C (anti-SYRGL) T cells in vitro only in the presence of dendritic cells led to the examination of the effect of
 25 hsp65-P1 on dendritic cells directly. As shown in Fig. 10A, when immature bone-marrow derived dendritic cells (day 6 in culture) were incubated overnight with various concentrations of hsp65-P1 the dendritic cell surface level of an MHC class I molecule (K^b) was increased. The extent of the increase depended on the hsp65-P1 concentration, and no increase was seen when hsp65-P1 was replaced by a series of control proteins

and peptides (hsp65 alone, P1 alone, SYRGL, Mal-P1, or a monoclonal IgG antibody [anti-2,4,6, trinitrophenyl]).

Other hsp65 fusion proteins, having various fusion partners (influenza virus nucleoprotein or the E7 subunit of human papilloma virus) also elicited increased
5 expression of K^b on the dendritic cells (Fig. 10A). It is important to note, however, that unmodified hsp65 ("hsp65 only" in Fig. 10A, 10B) consistently failed to stimulate dendritic cell upregulation of K^b.

All of the fusion proteins as well as unmodified hsp65 were produced as recombinant proteins in *E. coli* and contained trace levels of endotoxin
10 (lipopolysaccharide, LPS). An endotoxin standard by itself evoked a weak response at the highest concentration tested (5 EU/ml, Fig. 10B). Because of mol. wt. heterogeneity of LPS, conversion of endotoxin units into LPS weight and mole units is highly approximate. But, if one EU corresponds to about 5 ng LPS, and the "average" mol. wt. of LPS is taken to be approximately 10,000, LPS would appear to be somewhat more
15 effective than hsp65 fusion proteins in activating dendritic cells. Nevertheless, the effects of the fusion proteins seemed clearly not to be due to endotoxin contaminants, because when hsp65-P1, hsp65, or Mal-P1 were each added in amounts that resulted in addition of equivalent EU units to the dendritic cells, increased expression of K^b was elicited only by hsp65-P1. Moreover, when the data from Fig. 10A were plotted against
20 the EU concentrations attributable to the controls and fusion proteins, it was evident that each of the four hsp65 fusion protein preparations, but none of the controls, stimulated increased expression of MHC class I protein. Finally, all the hsp65 fusion proteins elicited increases in MHC class I expression on dendritic cells from C3H/HeJ mice, a strain known to be unresponsive to LPS (due to a mutation in the Toll4 receptor)
25 (Poltorak, A., *et al.*, *Science*, 282:2085-2088 (1998)). Taken together, the findings demonstrate that activation of the dendritic cells was due to the hsp65 fusion proteins, not to endotoxin contaminants. Besides stimulating the dendritic cells (bone marrow derived and maintained in culture with GM-CSF for 6 days) to express increased levels of MHC class I, the hsp fusion proteins stimulated increased expression of MHC class II
30 and B7.2 (CD86) (the Table); the level of CD40 was, however, only marginally

affected. Native hsp65 did not affect expression of MHC class II or B7.2, just as it failed to affect levels of MHC class I.

Table

Heat shock fusion proteins stimulate increased expression of MHC and costimulatory

5 (B7.2) molecules on dendritic cells

	Nothing	unmodified hsp65	P1 peptide	hsp65-NP	hsp65-P1
MHC Class I	100	105	96	257	151
MHC Class II	63	67	55	279	90
B7.2	60	54	47	129	81
CD40	45	45	47	76	51

10 Dendritic cells from bone marrow of C3H/HeJ mice were incubated for 18 hrs with 1.5×10^{-6} M of various heat shock proteins or the control P1 peptide prior to cell surface staining.

Activated dendritic cells in vivo

That the dendritic cell changes could also be elicited in vivo was indicated by
 15 the finding that 24 hrs after injecting hsp65-P1 (in saline) subcutaneously into mice, myeloid dendritic cells (but not lymphoid dendritic cells) from lymph nodes draining the site of injection showed increased expression of K^b (Fig. 9B).

To determine if activated DC were especially effective in vivo, normal B6 mice were adoptively transferred with 2×10^6 naive 2C cells (from 2C TCR transgenic mice,
 20 see Cho, B., *et al.*, *Proc. Natl. Acad. Sci. USA*, 96:2976-2981 (1999)) and the next day the recipients were injected in a hind footpad with 8×10^5 dendritic cells. The dendritic cells had been incubated overnight with or without hsp65-NP (to generate activated or nonactivated dendritic cells, respectively), and then incubated for 2 hrs with SYRGL

peptide at various concentrations (0 , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} M) and washed just before the cells were injected. 24 hrs later 2C CD8⁺ T cells from the draining popliteal lymph nodes were examined for CD69 expression as evidence of having been antigenically stimulated. As shown in Fig. 9C, when the peptide concentration was 10^{-9} M, the activated dendritic cells were considerably more effective than the nonactivated dendritic cells in stimulating the naive 2C T cells to express CD69. When pulsed with the peptide at 10-1000-times higher concentrations activated and nonactivated dendritic cells were about equally effective, stimulating CD69 responses of the 2C CD8⁺ cells at about the level seen in Fig. 9C, bottom panel.

- 10 Stimulation of CD8 CTL production in vivo by the hsp65-P1 fusion protein does not require the participation of CD4 T cells

The ability of the hsp fusion proteins to directly stimulate dendritic cells suggested that CD4 T cells might not be necessary for the CD8 T cell response elicited in vivo by the fusion proteins. To test this possibility, CD4 knockout mice (CD4^{-/-}) were immunized using the same regimen as before (Fig. 6A-6C) and their ability to produce SYRGL-specific CTL was assessed. As seen in a representative response in Fig. 10C, the CD4^{-/-} mice produced CTL in response to hsp65-P1 but not in response to the control Mal-P1. While the cytolytic activity elicited in the CD4^{-/-} mice (n=6) was unambiguous, it appeared to be somewhat less than was generally elicited in normal C57BL/6 mice. In other experiments C57/BL6 mice that had been extensively depleted of CD4 cells by repeated injections or an anti-CD4 mAb (GK1.5) also responded to the standard immunization protocol with hsp65-P1 about as well as untreated normal mice (data not shown). All of these results show that in stimulating CD8 CTL production in mice hsp65-P1 does not require the participation of CD4 T cells.

25 Discussion

As shown herein, mycobacterial hsp65 fused to the P1 polypeptide activates dendritic cells and stimulates, in the absence of CD4⁺ T cells, the production of CD8⁺ CTL that recognize a short peptide derived from P1. The findings extend the number

and diversity of hsp fusion proteins that can elicit CD8 T cell responses and suggest a potential mechanism by which the fusion proteins exert their effects in the absence of added adjuvants, a prominent feature of the in vivo responses to all hsp fusion proteins. Generally, where CD4 T cell help and adjuvants are required for CD8 T cell responses, it is likely that they function by activating dendritic cells (Bennett, S., *et al.*, *Eur. J. Immunol.*, *Nature*, 393:478-480 (1998); Ridge, J., *et al.*, *Nature*, 393:474-478 (1998) and Schoenberger, S., *et al.*, *Nature*, 393:480-483 (1998)). It is reasonable to expect that the capacity of heat shock fusion proteins to directly activate dendritic cells accounts for their ability to bypass the requirements for CD4 T cells and added adjuvants.

HSP fusion proteins activate dendritic cells directly

Using CTL to detect polypeptide processing by APC, previous studies pointed to macrophages, or equally to macrophage and dendritic cells, as being responsible for processing protein immunogens that elicit CD8 T cell responses (Kovacs-
Bankowski, M., *et al.*, *Science*, 267:243-245 (1995); Rock, K., *Today*, 17:131-137 (1996); Suto, R. and Srivastava, P.K., *Science*, 269:1585-1588 (1995)). As shown herein, when macrophages and dendritic cells were incubated with hsp65-P1 they become equally susceptible to lysis by peptide-specific CTL in a standard 4 hr cytolytic assays, indicating that both types of APC could generate small peptides from the hsp fusion protein and load them on the MHC class I molecules. However, when these cells were evaluated for their ability to stimulate naive CD8 T cells to proliferate and produce IL-2 and IFN- γ , the dendritic cells, but not the macrophages, proved to be effective. A step towards understanding this difference comes from the present finding that hsp65-P1, as well as each of the other hsp65 fusion proteins tested, is capable of directly stimulating dendritic cells to increase their surface expression of MHC class I and II and costimulatory (B7.2) molecules.

Dendritic cells infected with mycobacteria, including BCG, or streptococci, or *Leishmania* have been shown to upregulate MHC and costimulatory molecules B7.1 and B7.2 and, in addition, to secrete IL-12 (Demangel, C., *et al.*, *Eur. J. Immunol.*, 29:1972-

1979 (1999); Henderson, R., *et al.*, *J. Immunol.*, 159:635-643 (1997); Konecny, P., *et al.*, *Eur. J. Immunol.*, 29:1803-1811 (1999); Rescigno, M., *et al.*, *Proc. Natl. Acad. Sci. USA*, 95:5229-5234 (1998)). It may be that microbial cell hsp molecules are responsible for these effects. If so, the findings described herein (of a difference between hsp65 fusion proteins and unmodified hsp65) indicate that upregulation of these activation molecules are due to the hsp in a modified form, resembling perhaps the hsp65 fusion proteins studied here, rather than naive hsp molecules.

Dendritic cells infected with certain viruses, e.g., influenza virus (Ridge, J., *et al.*, *Nature*, 393:474-478 (1998)), likewise become activated. However, the hsp fusion proteins appear, so far, to be the only soluble immunogenic proteins that directly activate dendritic cells, in vitro and in vivo, to upregulate expression of MHC and costimulatory molecules. The experimental system described herein is useful for investigating the pathways by which hsp fusion proteins are processed and presented by dendritic cells and the mechanisms by which MHC and costimulatory molecules are up-regulated.

Hsp fusion protein stimulation of CD8 T cell production does not depend upon CD4 T cells

Prior to the present study one way to account for the ability of hsp fusion proteins to stimulate CD8 T cell production was to invoke a key role for CD4 T cells. Thus, a vigorous CD4 T cell response to peptides from the hsp moiety could activate dendritic cells and amplify an otherwise marginal CD8 T cell response to peptides from the fusion partner (Bennett, S., *et al.* *Nature*, 393:478-480 (1998); Ridge, J., *et al.*, *Nature*, 393:474-478 (1998); Schoenberger, S., *et al.*, *Nature*, 393:480-483 (1998)). This possibility is supported by older evidence that hsp65 can serve as an effective carrier molecule in the classic sense: i.e., when chemically coupled to nonimmunogenic hapten-like molecules (polysaccharides, a malarial peptide) the conjugates elicited IgG antibodies to the adducts in responses that were presumably T-cell dependent (Barrios, C., *et al.*, *Eur. J. Immunol.*, 22:1365-1372 (1992)). This mechanism is clearly not essential, because CD4^{-/-} mice injected with hsp65-P1 produced cytolytic CD8 T cells

to the fusion partner's peptide. Nevertheless, it is entirely possible that in normal animals the response may be enhanced by CD4 T cells specific for peptides derived from the hsp moiety.

Previous efforts to determine whether CD4 T cells are essential for CD8 T cell responses to various immunogens and immunization strategies have yielded diverse results. With some epitopes, e.g., minor histocompatibility antigens, CD8 T cell responses could not be elicited in CD4^{-/-} mice (DiRosa, F. and Matzinger, P., *J. Exp. Med.*, 183:2153-2163 (1996)), but with more potent immunogens, (e.g., lymphocytic choriomeningitis virus or a murine herpes virus), or high doses of particulate antigens, CD8 CTL responses in CD4^{-/-} mice were virtually the same as in normal mice (Rahemtulla, A., *et al.*, *Nature*, 353:180-184 (1991); Rock, K. and Clark, K., *J. Immunol.*, 156:3721-3726 (1996); Stevenson, P., *et al.*, *Proc. Natl. Acad. Sci. USA*, 95:15565-15570 (1998)). That CD4 T cells are not required for the CD8 T cell response to hsp65-P1 indicates that hsp fusion proteins are relatively potent immunogens for CD8 T cells.

The hsp moiety in hsp fusion proteins

In the several hsp fusion proteins examined here the only common element is hsp65. The question arises as to how the hsp moiety can directly activate dendritic cells (and thereby elicit CD8 CTL production), regardless of wide variations in length and sequence of the fusion partners. It is particularly notable, in contrast, that unmodified ("native") hsp65 lacks this critical activity. It may be that in the fusion proteins the hsp moiety adopts a particular conformation or displays a linear sequence or peptide motif or pattern that is i) necessary for eliciting the dendritic cell response, ii) retained despite wide variations in the fusion partner sequences, and iii) absent or masked in unmodified ("native") hsp65.

The intensity of current interest in CD8 vaccines for HIV-1 and other persistent intracellular pathogens, as well as for cancer cells, is reflected in recent studies of diverse genetic vaccines and of several bacterial toxins fused to antigenic peptides or polypeptides as stimulators of CD8 CTL production. For example, nonapeptide

sequences inserted into a truncated subunit of anthrax toxin or pertussis toxin, could elicit CD8 CTL in vivo (Ballard, J., *et al.*, *Proc. Natl. Acad. Sci. USA*, 93:12531-12534 (1996) and render target cells susceptible to lysis by cognate CD8 CTL in vitro (Goletz, T., *et al.*, *Proc. Natl. Acad. Sci. USA*, 94:12059-12064 (1997); Carbonetti, N., *et al.*,
5 *Infect. Immun.*, 67:602-607 (1999)). These and other bacterial toxins have evidently acquired through evolution the capacity to cross mammalian cell membranes and gain access to the cell cytosol, where they exert their lethal effects. While the judicious linkage of small peptides allows these toxin subunits to retain their ability to traverse
10 membranes, the need to preserve this special property may limit the size and sequence diversity of the fusion elements that can be accommodated. For the hsp fusion proteins, in contrast, there appear so far to be no constraints to their effectiveness as CD8 immunogens by the length or sequence of the fusion partners. Because large fusion
partners, e.g., the equivalent of a typical protein domain, are likely to encompass many potential epitopes for diverse MHC class I molecules, the hsp fusion proteins as a class
15 are candidate vaccines for use with populations of MHC-disparate individuals.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.